Adenosine A_{2B} receptor stimulates angiogenesis by inducing VEGF and eNOS in human microvascular endothelial cells

Xiaolong Du¹, Xuehai Ou², Tao Song², Wentao Zhang², Fei Cong², Shihui Zhang² and Yongmin Xiong¹

¹Institute of Endemic Diseases, Xi'an Jiaotong University Health Science Center, Key Laboratory of Trace Elements and Endemic Diseases, National Health and Family Planning Commission, Xi'an Jiaotong University, Xi'an Shaanxi 710061, China; ²School of Medicine, Xi'an Jiao Tong University, Xi'an Shaanxi 710061, China
Corresponding author: Yong-Min Xiong. Email: xiongyongmin@126.com

Abstract

Angiogenesis is critical to wound repair due to its role in providing oxygen and nutrients that are required to support the growth and function of reparative cells in damaged tissues. Adenosine receptors are claimed to be of paramount importance in driving wound angiogenesis by inducing VEGF. However, the underlying mechanisms for the regulation of adenosine receptors in VEGF as well as eNOS remain poorly understood. In the present study, we found that adenosine and the non-selective adenosine receptor agonists (NECA) induced tube formation in HMEC-1 in a dose-dependent manner. Adenosine or NECA (10 μ mol/L) significantly augmented the number and length of the segments in comparison with the control. Simultaneously, VEGF and eNOS were significantly upregulated following the administration of 10 μ mol/L NECA, while they were suppressed after A_{2B} AR genetic silencing and pharmacological inhibition by MRS1754. In addition, VEGF expression and eNOS bioavailability elimination significantly reduced the formation of capillary-like structures. Furthermore, the activation of A_{2B} AR by NECA significantly increased the intracellular cAMP levels and concomitant CREB phosphorylation, eventually leading to the production of VEGF in HMEC-1. However, the activated PKA-CREB pathway seemed to be invalidated in the induction of eNOS. Moreover, we found that the elicited PI3K/AKT signaling in response to the induction of NECA assisted in regulating eNOS but failed to impact on VEGF generation. In conclusion, the A_{2B} AR activation-driven angiogenesis via cAMP-PKA-CREB mediated VEGF production and PI3K/AKT-dependent upregulation of eNOS in HMEC-1.

Keywords: Adenosine A_{2B} receptor, angiogenesis, VEGF, eNOS, CREB

Experimental Biology and Medicine 2015; 240: 1472–1479. DOI: 10.1177/1535370215584939

Introduction

Traumatic digit amputations are one of the occupational injuries that often occur in young productive patients, representing 1% of all trauma attendances.¹ Microvascular replantation and neurovascular remodeling play pivotal roles in the structural and functional reconstruction of the replanted digits and wound healing and repair of the damaged tissues. In the past decades, more effort has been dedicated to understanding the formation of new blood vessels from the preexisting vasculature via angiogenesis, which is indispensable for successful ischemic wound restoration.² The early stages of angiogenesis proceed with capillary formation and the initiation of sprouting into the wound bed by endothelial cell proliferation and migration in response to diverse cytokines and metabolic stimulus. Vascular endothelial growth factor (VEGF) and nitric oxide (NO) are acknowledged to be the two key

regulators of normal and pathological angiogenesis.³ VEGF, released immediately after injury, is of paramount importance in driving wound angiogenesis. It can stimulate proliferation and migration of vascular endothelial cells and increase the permeability of vessels as well as the differentiation of endothelial cells into new capillary tubes.^{4,5} Furthermore, genetic deletion of endothelial NO synthase (eNOS) has been shown to result in the impairment of angiogenesis in a mouse model of hindlimb ischemia, and NOS2 has been identified as being involved in angiogenic signaling in the healing of skin wounds.⁶

Adenosine receptors (AR), including A₁, A_{2A}, A_{2B}, and A₃, are expressed in multiple tissues and cells, including endothelial cells in a tissue-dependent pattern, and this differential expression of adenosine receptor isoforms contributes to the functional heterogeneity of human endothelial cells. Among all the ARs, A_{2B} has been observed to be

preferentially expressed in human microvascular endothelial cells (HMEC).⁷ Simultaneously, emerging evidence has extended the role of AR to angiogenesis during wound healing and even tumor growth. The activation of AR, either by adenosine or the corresponding agonist, can drive physiologic and pathologic angiogenesis by suppressing antiangiogenic factor production while enhancing angiogenic factor production.⁸ An *in vivo* study found that an intravenous infusion of adenosine can increase plasma levels of VEGF in humans, despite being performed in a relatively small population.⁹ In addition, A_{2B} AR activation is implicated in the promotion of angiogenesis and release of VEGF from HMEC, while it has been clarified that A_{2A} AR stimulates VEGF gene expression in response to the hypoxia-induced accumulation of adenosine in retinal vascular cells.^{7,10,11} Moreover, it is validated that adenosine activates eNOS via A_{2A} AR in the nucleus tractus solitarius of rats, while NOS mediates A_{2B} AR-induced renal vasodilation in female rats.^{12,13}

Although the role of A_{2B} AR in angiogenesis has been subjected to studies, the underlying mechanism for the regulation of A_{2B} AR on VEGF secretion and NOS activation is poorly revealed. In the current study, we sought to evaluate whether A_{2B} AR is responsible for the modulation of VEGF and NOS, and then investigate the potential signaling pathways involving their regulation, which will facilitate the development of new strategies for the treatment of traumatic digit replantation and restoration of damaged tissues.

Materials and methods

Cell culture and treatment

HMEC-1 cells were obtained from the American Type Culture Collection and cultured as recommended in MCDB131 medium (GIBCO, Grand Island, NY) containing 10 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 g/ml streptomycin), and 10% FBS at 37°C in a humidified 5% CO₂ incubator. Cells were pretreated with 1 U/ml of adenosine deaminase (Calbiochem) to remove endogenously produced adenosine. Exogenous adenosine or adenosine receptor agonist NECA was purchased from Sigma-Aldrich and applied at a concentration ranging from 0.1 to 20 μmol/L and 0.1 to 100 μmol/L, respectively. For the analysis of cellular signaling cascades on the modulation of VEGF and eNOS, the HMEC-1 cells were incubated with cAMP activator 8CPT-2Me-cAMP, PKA inhibitor H-89 (10 μM) or LY294002 (a specific PI3K inhibitor) for 2 h before the addition of NECA.

Tube formation assay

Endothelial cell tube formation assay is a useful indicator of angiogenesis potential. Proliferation and migration of endothelial cell in response to diverse cytokines and metabolic stimulus drive the capillary formation and the initiation of sprouting, which are hallmark of early angiogenesis. In the current study, tube formation assay was performed as previously described.¹⁴ In brief, HMEC-1 cells (1.5 × 10⁴ cells per well) were seeded onto a 96-well plate pre-coated with

Matrigel (50 μl) and cultured under normal culture conditions. At the same time, various concentrations of adenosine (0.1–20 μmol/L) or NECA (0.1–100 μmol/L) were added to the cell cultures and incubated for 24 h. After being fixed with 70% ice-cold ethanol, the formed network of tubes were visualized at 100 × magnification by light microscopy. The total number and length of tubes following different treatments were analyzed by Image-Pro Plus 6.0 software (Rockville, MD, USA). Each individual experiment was performed in triplicate.

RT-PCR

RT-PCR was performed to investigate the expression of VEGF and eNOS. HMEC-1 cells were separately incubated with 10 μmol/L CPA (A_{2A} AR selective agonist), 10 μmol/L NECA, and 10 μmol/L MRS1754 (A_{2B} AR inhibitor) for 24 h. Total RNA was isolated and purified using an RNeasy Plus Universal Kit, according to the manufacturer's instructions (Qiagen, Santa Clara, CA, USA). RNA (1.5 μg) was reverse transcribed into cDNA that was utilized as a template in the subsequent RT-PCR amplifications using a SYBR Green Real-Time PCR Master Mix kit (Takara Biotechnology, Dalian, China). VEGF primers (sense 5'-CTACCTCCAC CATGCCAAGT-3'; anti-sense 5'-GCAGTAGCTGCGCT GATAGA-3') and an eNOS primer (sense 5'-TTCCG GCTG-CCACCTGATCCTAA-3'; anti-sense 5'-AACATA TGTCCTTGC-TCAAGGCA-3') were used, with GAPDH primers (sense 5'-GCCAAAAGGGTCATCATCTC-3'; anti-sense 5'-GTAGAGGCAGGGATGATGTTTC-3') as the internal control (Shanghai Biological Engineering Co., Ltd, China). The amplification cycles were as follows: 94°C for 3 min, then 33 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min, followed by 72°C for 15 min. Aliquots of the PCR products were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining. Real-time PCR experiments for each gene were performed on three separate occasions.

siRNA transfection

The construction of siRNA corresponding to the A_{2B} AR gene coding region was performed, as described previously.¹⁵ The A_{2B} AR siRNA sense and antisense sequences were 5'-ACCTCAACCGAGACTTCCGCTACACTTCAA GAGAGTGTAGCGGAAGTCTCGGTTTT-3' (sense) and 3'-CAAAAAAACCGAGACTTCCGCTACACTCTCTTGAAGT GTAGCGGAAGTCTCGGTTG-5' (antisense). The VEGF siRNA sense and antisense sequences were: 5'-CCA ACAUCACCAUGCAGAUdTdT-3' (sense) and 5'-AUCU GCAUGGUGAUGUUGGdTdT-3' (antisense). All of the siRNA duplexes were provided by Sangon Biotech Co. Ltd. The HMEC-1 cell line was seeded in a six-well plate at 5 × 10⁵ cells per well, grown for 24 h, and then transfected with 200 nmol/L A_{2B} AR siRNA using oligofectamine and RPMI-1640 reduced serum medium (Invitrogen Life Technologies Inc., Carlsbad, California, USA) according to the manufacturer's instructions. Silencing was measured 48 h after the transfection. Control cells were treated with oligofectamine carrying scrambled siRNA and serum-reduced medium (mock).

Figure 1 Effects of adenosine and NECA on the tube formation of HMEC-1. HMEC-1 cells were seeded in a 96-well plate pre-coated with Matrigel and were stimulated with a series of A_{2B} receptor agonist adenosine and NECA for 24 h. Representative light microscopy images of tube formation and tubule number (A, C) and length (B, D) were determined after the stimulation of adenosine and NECA. Data are presented as mean $\pm$ standard error of the mean based on three independent experiments. Bar = 200 μ m; * P < 0.05 compared with control in the absence of either adenosine or NECA

ELISA

Secreted VEGF was detected from the supernatant of cell cultures using Human VEGF Quantikine ELISA Kit (R&D Systems Europe Ltd., Oxon, UK) according to the manufacturer's protocols.

Western blot

For analysis of the expression of eNOS, CREB, pCREB, Akt, and pAKT (S473) in response to various treatments using Western blot, and total cell lysates were subjected to SDS-PAGE and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. Immunodetection was performed by the incubation with antibodies to eNOS (Millipore Corporation, Billerica, MA, USA), CREB/pCREB

(S133), and AKT/pAKT (S473) (Cell Signaling Technology, MA, USA). Labeled proteins were visualized using Amersham Western blot detection reagent (GE Healthcare).

Determination of cAMP concentration

The total concentration of cAMP in HMEC-1 cells was evaluated using cAMP Biotrak Enzyme Immunoassay (Amersham Biosciences, Piscataway, NJ, USA). A total of 2.5×10^5 HMEC-1 cells were seeded in 96-well plates and incubated with NECA, MRS 1754 or forskolin (10 μ M for each reagent) overnight at standard culture conditions (37°C, 5% CO₂), and cAMP determination was performed according to the manufacturer's protocols.

Figure 2 A_{2B} receptor is involved in the NECA-induced expression of VEGF and eNOS. RT-PCR analysis of VEGF (A) and eNOS (C) mRNA in response to pre-treatments with NECA (10 μmol/L), alone or combined with A_{2B} receptor siRNA and its antagonist MRS1754 as well as A_{2A} agonist CPA. ELISA and Western blotting were performed to determine the secreted VEGF (B) and expression of eNOS (D), respectively. VEGF antibody or siRNA and eNOS inhibitor L-NAME reduced tubule number (E) and length (F) in HMEC-1. Data are presented as mean ± standard error of the mean based on three independent experiments, **P* < 0.05, ***P* < 0.01 compared with control in the absence of NECA (10 μmol/L). #*P* < 0.05, ##*P* < 0.01 compared with NECA-treated HMEC-1 transfected A_{2B} receptor siRNA

Statistical analysis

Data are presented as mean ± standard error of the mean. Differences were compared by analysis of variance followed by Bonferroni test. A *P* value < 0.05 was considered statistically significant. All statistical tests were performed using SPSS, v. 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Adenosine and NECA increase tube formation of HMEC-1

To investigate the effects of adenosine receptor activation on angiogenesis, various doses of non-selective adenosine receptor (A_{2A}/A_{2B}) agonists, including adenosine and NECA, were added to the HMEC-1 cell cultures and the formation of capillary-like structures were determined. As shown in Figure 1(A) and (B), the number of segments and segment lengths were significantly increased in response to 10 μmol/L adenosine and 100 μmol/L adenosine had

comparable effects, while 0.1 and 1.0 μmol/L adenosine had no obvious impact on tube formation. Similarly, NECA also induced the increase of capillary-like structures in a similar manner as adenosine, and 10 μmol/L significantly augmented the number and length of the segments in comparison with the control (Figure 1(C) and (D)).

A_{2B} AR is involved in NECA-induced upregulation of VEGF and eNOS

VEGF and eNOS are two key determinants in angiogenesis; their expression in response to NECA was evaluated by RT-PCR and ELISA as well as western blotting, respectively. VEGF and eNOS were significantly upregulated following the administration of 10 μmol/L NECA, while A_{2A} AR selective agonist CPA failed to enhance the expression of VEGF and eNOS. The increased VEGF and eNOS induced by NECA were mitigated after the silencing of A_{2B} AR in HMEC-1 cells by the blockade of A_{2B} AR antibody and the administration of A_{2B} AR inhibitor MRS1754; therefore,

Figure 3 cAMP-PKA-CREB pathway is account for the upregulation of VEGF. (A) Accumulation of cAMP induced by the activator Forskolin, NECA or MRS 1754, alone and in combination, in HMEC-1 cells and A_{2B} receptor siRNA transfected HMEC-1. (B) Western blotting analysis of pCREB/CREB after incubation with NECA, MRS1754 as well as PKA inhibitor H89. VEGF (C) and eNOS (D) levels after incubation with cAMP activator 8CPT-2Me-cAMP and H89. Data are presented as mean $\pm$ standard error of the mean based on three independent experiments, * P < 0.05, ** P < 0.01 compared with control in the absence of NECA (10 μ mol/L). # P < 0.05, ## P < 0.01 compared with NECA-treated HMEC-1 cells

NECA-induced upregulation of VEGF and eNOS was mediated by A_{2B} AR rather than A_{2A} AR (Figure 2(A-D)). In addition, the inhibition of VEGF after the transfection of VEGF siRNA and incubation with VEGF antibody or

blockage of eNOS with L-NAME (100 μ M, 24 h) significantly reduced the NECA-induced tube formation of HMEC-1 as reflected in the decreased number and length of the capillary-like structures in Figure 2(E) and (F).

Figure 4 PI3K-AKT cascade is involved in the induction of eNOS. (A) Western analysis and quantification of pAKT (S473) in response to the treatments with NECA and MRS 1754. (B) Inhibition of PI3K-AKT signaling on the expression of eNOS was evaluated using western blotting. * $P < 0.05$ compared with control in the absence of NECA (10 $\mu\text{mol/L}$). # $P < 0.05$, ## $P < 0.01$ compared with NECA-treated HMEC-1 cells

cAMP-PKA-CREB pathway is involved in the regulation of VEGF in HMEC-1 cells

To determine whether A_{2B} AR is implicated in the regulation of intracellular cAMP, we evaluated the concentration of cAMP in HMEC-1 cells. The results showed that 10 $\mu\text{mol/L}$ Forscolin (a well-established inducer of cAMP) significantly increased the accumulation of cAMP; likewise, NECA also significantly enhanced the production of cAMP compared with the control ($P < 0.01$). The incubation of A_{2B} AR inhibitor MRS1754 or the introduction of A_{2B} AR siRNA significantly attenuated intracellular cAMP induced by NECA in HMEC-1 cells (Figure 3(A)). We then assessed the effects of its downstream PKA-CREB signaling on the modulation of VEGF and eNOS. Compared to the untreated control, pCREB/CREB was significantly elevated in the presence of NECA while reduced following treatment with MRS1754. At the same time, PKA blockage with H-89 (10 μM) significantly abrogated the NECA-induced phosphorylation of CREB (Figure 3(B)). We observed the regulation of cAMP-PKA-CREB signaling on the expression of VEGF and eNOS using ELISA and western blotting, respectively, as shown in Figure 3(C), cAMP activator 8CPT-2Me-cAMP further enhanced the expression of VEGF compared to the sole incubation of NECA. H-89 significantly suppressed the NECA-induced VEGF overexpression ($P < 0.05$). However, the upregulation of eNOS stimulated by NECA did not significantly change either in the presence of 8CPT-2Me-cAMP or H-89 (Figure 3(D)).

PI3K/AKT signaling accounts for the regulation of eNOS

NECA-induced upregulation of eNOS is independent of the PKA-CREB cascade; therefore, we intended to clarify whether PI3K/AKT signaling mediated the induction of NECA on eNOS. We found that NECA triggered the

PI3K/AKT pathway by promoting the phosphorylation of AKT (S473), while MRS1754 inhibited the presentation of pAKT (S473) induced by NECA (Figure 4(A)). Moreover, eNOS significantly diminished in response to the culture compared to NECA as the sole treatment (Figure 4(B)).

Discussion

Angiogenesis is initiated through a convergence of diverse signaling mechanisms involving prominent pathways that include the autocrine effects of eNOS-derived NO and VEGF.¹⁶ Moreover, AR have attracted attention for their contribution to angiogenesis. However, the underlying mechanism regarding the modulation of AR on the secretion of VEGF and NO remain unrevealed. Our study has suggested that A_{2B} is responsible for the onset of angiogenesis by synergistically activating cAMP-PKA-CREB-mediated VEGF and PI3K/AKT-mediated eNOS.

A_{2B} AR subtype displays low affinity with the endogenous agonist compared with the A₁, A_{2A}, and A₃ subtypes, and is activated in response to the higher concentrations of adenosine following tissue damage such as ischemia and inflammation.¹⁷ In this work, we also found that A_{2B} AR agonists at lower concentration than 0.1 or 1 μM are unable to obviously stimulate the tube formation *in vitro*. Therefore, the A_{2B} AR represents a potential target for pharmacological repair of damaged tissues. Adenosine is an important endogenous purine nucleoside and acts as a metabolic sensing molecule, which is currently utilized in the clinical treatment of supraventricular tachycardia or as a coronary vasodilator during radionuclide myocardial perfusion imaging.¹⁸ Related data has revealed that adenosine is produced following the induction of hypoxia and ischemia and accumulated at the sites of tissue injury,

inflammation, and local hypoxia.^{10,19} Moreover, it is well-established that VEGF is induced when the tissue is subjected to hypoxia and ischemic attack. The association of adenosine and VEGF secretion dependent on A_{2A} or A_{2B} AR is also documented in multiple studies.^{20,21} In this study, we found that A_{2B} rather than A_{2A} AR is implicated in the adenosine and NECA elicited upregulation of VEGF, based on the observation that an A_{2A} AR agonist CPA exerted a negligible effect on the expression of VEGF. In addition, A_{2B} has been observed to be preferentially expressed in human microvascular endothelial cells. Simultaneously, activation of A_{2B} AR also promotes eNOS expression, which is involved in angiogenesis by triggering NO synthesis and stimulating the central EGF-R signaling pathway.²²

A_{2B} AR belongs to the G protein-coupled receptor family and is clarified as a G_s protein that accounts for the increase of classical intracellular second messenger cAMP. It has been demonstrated that A_{2A} and A_{2B} AR activate the cAMP/PKA pathway and mediate TSP-1 production in macrophages.²³ In addition, activation of A_{2B} receptors promotes the phosphorylation of CREB in trophoblast cells.²⁴ In the current study, we identified that A_{2B} receptors elicit the increase of the intracellular cAMP level, then PKA is activated and diffuses into the nucleus to phosphorylate CREB. Moreover, the transcription factor pCREB contributed to the expression of VEGF, as demonstrated by the pharmacological (Ado agonists and antagonists) and genetic (siRNA) tools, which is in accordance with the previous reports that the PKA/CREB pathway is closely involved in VEGF expression in mouse macrophages.²⁵ Additionally, a more recent study has revealed that A_{2B} AR-dependent stimulation of VEGF production is attributed to the JunB binding to the VEGF promoter in HMEC-1 cells.¹⁰ Therefore, the cAMP-PKA-CREB cascade represents one of the vital signaling pathways in A_{2B}-mediated angiogenesis, and further investigation of this is needed. Furthermore, we found that the suppression of PKA or activation of cAMP did not alter the expression of eNOS, indicating that cAMP-PKA-CREB was not indispensable to the modulation of eNOS. We then focused on the PI3K/AKT pathway in the regulation of eNOS; the results showed that the pAKT/AKT increased when the A_{2B} AR was activated and the phosphorylation of AKT facilitates the expression of eNOS. An interesting study has reported that increased angiogenesis in HUVEC-C are mediated by the PI3K-Akt-eNOS signal pathway following simulated microgravity.²⁶

Above all, the present study showed that A_{2B} participated in the adenosine or NECA-induced angiogenesis in HMEC-1 cells by upregulating VEGF and eNOS, and cAMP-PKA-CREB is responsible for the modulation of VEGF, while A_{2B} mediated eNOS expression is dependent of the activation of PI3K-AKT signaling. However, the angiopreventive potential of the crosstalk of cAMP-PKA-CREB-VEGF and PI3K-AKT-eNOS in response to A_{2B} warrants further investigation.

Author contributions: XLD wrote the manuscript and participated in the design and interpretation of the studies;

XHO, TS, and WTZ conducted the experiments; FC and SHZ analyzed the data; YMX participated in the design and review of the manuscript.

ACKNOWLEDGEMENTS

The work was supported by grants from National Science Foundation of China (No.81172610).

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(Received January 29, 2015, Accepted March 31, 2015)